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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
A01N 43/08, A61K 31/365, C02F 1/50	A1	WO 99/27786
		(43) International Publication Date: 10 June 1999 (10.06.99)

(21) International Application Number: PCT/GB98/03548

(22) International Filing Date: 26 November 1998 (26.11.98)

(30) Priority Data:
9725599.6 4 December 1997 (04.12.97) GB

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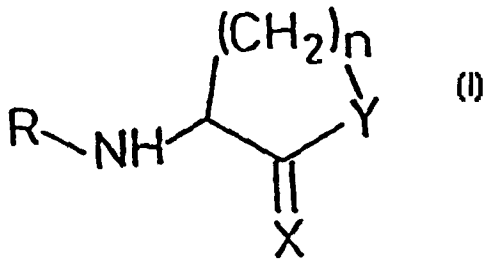
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CONTROL OF BIOFILM FORMATION

(57) Abstract

Compounds of formula (I) wherein n is 2 or 3; Y is O, S or NH; X is O, S or NH; and R is C_1–C_{18} alkyl or acyl, which may be substituted, may be used in the treatment or prevention of a bacterial infection in humans or animals by control of colonisation. The compounds may also be employed to remove biofilms from surfaces and are therefore useful in antibacterial articles and compositions.



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CONTROL OF BIOFILM FORMATION

This invention relates to the control of biofilm formation. In one aspect, the invention relates to the use of a group of compounds for treating and/or preventing bacterial infections in humans or animals by control of biofilm formation. The invention also relates to a method of removing a biofilm from a surface, to antibacterial compositions and to articles coated and/or impregnated with a compound which inhibits and/or prevents biofilm formation.

The treatment and prevention of bacterial infections are important in many different areas. For example, in seawater culture of salmonids, the bacterial infections vibriosis and furunculosis are the most important diseases in many parts of the world. Cold-water vibriosis is also of great significance in Atlantic salmon in regions with low water temperatures. Disease-control is possible by good husbandry practices, disease-resistance stock, improved diets, non-specific immunostimulants, antimicrobial compounds and vaccines. Current procedures are not without limitations however, for example there are problems of temporary immunosuppression following vaccination against *Aeromonas salmonicida* infection. Empirical observations have indicated that immunosuppression persists for some time after vaccination, rendering fish, especially subclinical carriers of *A. salmonicida*, highly vulnerable to bacterial invasion. The use of antibiotics such as amoxycillin as a control measure for furunculosis may contribute significantly to the spread of multiple antibiotic resistance in bacteria, and because of growing concern over the impact of these on the clinical treatment of disease, the use of such antibiotics in agriculture is likely to be highly restricted by future

legislation. Thus, the development of significant improvements in vibriosis and furunculosis disease control remains a major priority in aquaculture. A new understanding of the molecular biology of bacterial cell-cell communication has identified a novel method for the control of *Aeromonas* spp and *Vibrio anguillarum*, key targets in vibriosis and furunculosis disease control.

In recent years it has become evident that many different Gram-negative bacteria employ *N*-acylhomoserine lactones (AHLs) as diffusible signal molecules ("pheromones") as part of a cell-cell communication system that facilitates the induction of genetic regulons only when a significant population of cells has accumulated. Now termed "quorum sensing", this signal transduction pathway is involved in the regulation of diverse physiological processes including bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer and the production of exoenzyme virulence determinants in animal and plant pathogens (Salmond et al., Mol. Microbiol. 16 : 615-624, 1995; Swift et al., Trends Biochem Sci. 21 : 214-219, 1996). The archetypal model for quorum sensing is the *lux* operon which confers a bioluminescent phenotype on *Photobacterium (Vibrio) fischeri*. Light emission in *P. fischeri* is regulated at the transcriptional level via the LuxR and LuxI proteins. LuxR is a transcriptional activator which responds to the pheromone *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), the biosynthesis of which is dependent on the function of the *luxI* gene product (Sitnikov et al., Mol.Microbiol. 17, 801-812, 1995). In other Gram-negative bacteria, a family of LuxRI homologues has now been identified alongside a "molecular language" of AHLs which vary predominantly in the

presence or absence of an acyl chain C3 substituent (oxo-or hydroxy-) and length of the *N*-acyl side chain.

An indication of a role for AHLs in pathogenesis has come from studies of the opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* secretes many extracellular toxic factors including exotoxins and various tissue damaging exoenzymes including alkaline protease and elastase. All of these virulence determinants are regulated via quorum sensing (Williams et al., In Molecular biology of Pseudomonads, Eds. Nakazawa et al, p. 195-206, ASM Press, Washington, USA, 1996). Thus far, two LuxR homologues (LasR and VsmR[RhlR]) and two LuxI homologues (LasI and VsmI) have been described in *P. aeruginosa* PAO1 and their cognate signal molecules *N*-(3-oxododecanyl)-L-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL) chemically characterized. Both regulatory loci are involved in the expression of alkaline protease and elastase.

Spent culture supernants from both *Aeromonas hydrophila* and *Aeromonas salmonicida* activate a range of biosensors responsive to AHLs. The genes for a quorum sensing signal generator and response regulator have been cloned and termed *ahyRI* and *asaRI* respectively. Protein sequence homology analysis placed the gene products within the family of LuxRI homologues (Swift et al., J Bacteriol. 197, 5271-5281, 1979). *N*-(butanoyl)-L-homoserine lactone (BHL) is identified as the major AHL synthesized. When introduced into *E. coli*, both AhyI and AsaI direct BHL synthesis.

Transcriptional reporter studies with *ahl::luxCDABE* fusions indicate that AhyR and BHL are required for *ahyI* transcription. The serine

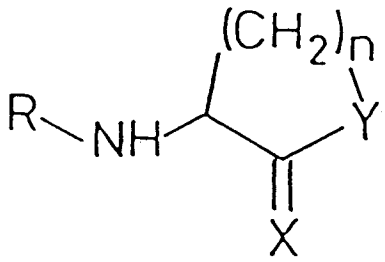
protease activity of *A. salmonicida* has been identified as a phenotype controlled by the *asaRI* quorum sensing regulon. Provision of exogenous BHL increases final levels of serine protease activity and promotes its earlier induction during the growth phase. When either *N*-decanoyl-L-homoserine lactone (DHL) or *N*-(3-oxohexanoyl)-L-homoserine lactone (ODHL) is added, the final activity of the serine protease is reduced. ODHL also delayed the induction of serine protease during growth from A_{650} 0.9 to A_{650} 1.2.

Many species of bacteria require the formation of a biofilm in order to colonise a surface, for example during the early stages of infection. The biofilm comprises the bacterial cells embedded within a sticky matrix of a mucoid substance known as "slime". It is now widely accepted that following the initial attachment phase, the second stage of infection by bacteria involves slime production and biofilm formation. The slime typically consists mainly of polysaccharides with about 10-20% proteins and probably stabilises the biofilms by promoting bacterial cell-to-cell and cell-to-surface associations so that multi-layered cell clusters accumulate on the infected surface. This sticky matrix helps the bacteria to survive, allowing them to feed and, where the surface is on a living human or animal, to interfere with host cellular defences and antibody production. Once formed, biofilms can be difficult to remove from a surface.

Unexpectedly, we have now found that quorum sensing is a major regulator of biofilm control and that quorum sensing blockers can therefore be used to prevent and/or inhibit biofilm formation. Also, the quorum sensing blockers are effective in removing, or substantially decreasing the amount of, biofilms which have already

formed on a surface. This is a new approach to dealing with bacterial infections.

Accordingly, the present invention provides the use of a compound of formula (I):



(I)

wherein: n is 2 or 3

Y is O, S or NH

X is O, S or NH

and R is C_1-C_{18} alkyl or acyl which may be substituted,

in the manufacture of a medicament for the treatment and/or prevention of a bacterial infection in humans or animals by control of colonisation. A key feature of the control of colonisation is the control of biofilm formation.

The invention also contemplates a method for treating and/or preventing a bacterial infection in a human or an animal comprising administering to the human or animal a therapeutically effective amount of a compound of formula (I).

For a given type of bacteria, the precise compound or compounds of formula (I) which is/are suitable for treating and/or preventing the

infection by controlling biofilm formation can be readily determined by the skilled person using nothing more than routine experminentation based on trial and error. For example, although a given compound of formula (I) may act as a quorum sensing molecule for a certain strain of bacteria, it may act as a quorum sensing blocker for other strains. It is the compounds which act as quorum sensing blockers in a given strain of bacteria which inhibit or prevent biofilm formation by that strain and, therefore, it is these compounds which are useful in the treatment of infection by that bacterium. As mentioned above, the identification of a suitable compound for treating and/or preventing infection by a given bacterium is a routine matter.

The preferred compounds of formula (I) are those in which Y is O, X is O, n is 2 and R is acyl. More preferably, R carries a keto or hydroxy group, suitably in the 3-position (i.e., the beta-position when R is acyl).

The term "alkyl" as used herein covers branched and unbranched, but preferably unbranched, alkyl groups, optionally substituted, preferably by an oxo or hydroxy group. The term "acyl" is defined in a corresponding manner.

The compounds used in the invention contain at least one chiral centre and they may be employed as a pure enantiomer, an optically active mixture of enantiomers or a racemic mixture.

Compounds of formula (I) in which R is a C_8-C_{18} group (such as a C_8 to C_{18} acyl group) have been found to be particularly effective as controllers of biofilm formation. For example, *N*-(3-oxodecanoyl)-L-

homoserine lactone (ODHL) significantly antagonises the formation of biofilms by *Aeromonas hydrophila*. The compounds with longer chain lengths than ODHL are believed to have increased potency. Particularly preferred are compounds having chain lengths of 12 to 14 carbon atoms, such as *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-(3-oxotetradecanoyl)-L-homoserine lactone; these longer chain compounds not only antagonise biofilm formation but also have the unexpected advantage of substantially abolishing protease production from certain wild type strains of *Aeromonas hydrophila*. A reduction in protease production, and particularly its abolition, helps to diminish the virulence of the bacteria.

It is known that AHL quorum sensing blockers can reduce protease production by 50% in some strains of bacteria but the discovery that certain compounds can substantially eliminate protease production imparts clear significant clinical advantages. Furthermore, the unexpected finding that biofilm formation can be inhibited or prevented by quorum sensing blockers leads to the reasonable conclusion that other AHL quorum sensing blockers which are known to exhibit quorum sensing blocking in other systems, such as protease production, will also be effective against biofilm formation.

The compounds of the invention are advantageously used to treat and/or prevent infections caused by *Vibrio anguillarum* or *Aeromonas* spp. Examples of this type of infection are vibriosis and furunculosis disease in fish. Inhibition of biofilm formation by the bacteria, optionally together with a reduction or elimination of extracellular protease production, renders the bacteria substantially non-pathogenic.

The compounds of the invetion may be formulated by conventional methods for use in the treatment and/or prevention of bacterial infection. For example, the compounds may be used as solid or liquid preparations (such as tablets, suspensions or solutions for oral administration or sterile injectable compositions), optionally together with pharmaceutically acceptable diluents, carriers or other additives. For the treatment of vibriosis or furunculosis disease in fish, the compounds or compositions containing them may be applied directly to the fish or they may be added to the fish's food or water.

In another embodiment, the invention provides a method of removing a biofilm from a surface which comprises treating the surface with a compound of formula (I). The surface is preferably the inside of an aqueous liquid distribution system, such as a drinking water distribution system or a supply line connected to a dental air-water system. The removal of biofilms from this type of surface can be particularly difficult to achieve. The compound is preferably applied to the surface as a solution of the compound either alone or together with other materials such as conventional detergents or surfactants.

A further embodiment of the invention is an antibacterial composition comprising a compound of formula (I) together with a bacteriocidal agent. In the antibacterial compositions, the compound of formula (I) helps to remove the biofilm whilst the bacteriocidal agent kills the bacteria. The antibacterial composition is preferably in the form of a solution or suspension for spraying and/or wiping on a surface.

In yet another embodiment, the invention provides an article coated and/or impregnated with a compound of formula (I) in order to inhibit

and/or prevent biofilm formation thereon. The article is preferably of plastics material with the compound of formula (I) distributed throughout the material.

The invention will now be described with reference to the following non-limiting examples.

EXAMPLES

Protease Assay

An inoculant of *Aeromonas hydrophila* A1[1] was grown overnight at 30°C in LB-broth, Lennox (Difco) containing 10μM of the specified AHL [*N*-(octanoyl)-L-homoserine lactone (OHL); *N*-(decanoyl)-L-homoserine lactone (DHL); *N*-(dodecanoyl)-L-homoserine lactone (dDHL); *N*-(3-oxodecanoyl)-L-homoserine lactone (ODHL); *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL); *N*-(3-oxotetradecanoyl)-L-homoserine lactone (OtDHL)], diluted 1:1000 from a 10mM stock solution in acetonitrile (Far UV HPLC grade). Cells were removed from the medium after incubation by micro-centrifugation, at a maximum speed, for 2 minutes and 50μl aliquots of supernatant were taken for assay. 500μl of 0.25% (w/v) azocasein (Sigma) was added to each supernatant aliquot to be tested and incubated at 37°C for 2 hours. 0.25% azocasein was prepared by dissolving azocasein (solid) at 0.5% (w/v) in 0.1M sodium citrate pH 6 and incubation in a boiling water bath for 2 to 5 minutes, diluting to 0.25% with 0.1M sodium citrate pH 6 and filtration through Whatman® No. 1 paper to remove any undissolved particles. The protease reaction was stopped, and protein precipitated, by the

addition of 550µl of ice cold 10% (w/v) trichloroacetic acid followed by incubation on ice for 15 minutes. Azodye released by the action of proteases in supernatant aliquots was measured by absorbance at 366nm (A_{366nm}) after the removal of precipitated protein by micro-centrifugation at maximum speed for 5 minutes. Relative protease activity (Table 1) is 1000x the A_{366nm} value. Errors reflect 1 standard deviation from the mean $n = 3$.

Table 1

TREATMENT	RELATIVE PROTEASE ACTIVITY
Untreated control	176±1.2
10µM DHL	16 + 1.5
10µM dDHL	62 ± 2.3
10µM ODHL	106 ± 1.7
10µM OdDHL	1 ± 0.6
10µM OtDHL	Zero

Biofilm Assay

Stainless steel coupons (type 304 with a 2B finish; Campden Food and Drink Research Association, Chipping Campden, UK) cut into 10mm squares, cleaned by scrubbing in a neutral detergent, rinsed with sterile water and sterilised at 140°C for 4 hours were used as biofilm substrata. For biofilm formation, sterile coupons were immersed in 5 ml 10% (v/v) L-broth inoculated to provide a 1:1000 dilution of an overnight bacterial culture. The coupons, media, and the AHL (final concentration 50µM) were contained in a 55mm sterile plastic petri dish and rotated at 70-90 rpm at 30°C. After the stated period of incubation (Table 2) coupons were washed with sterile water, samples were gently heat fixed in the flame of a Bunsen burner and stained by incubation in 40µl of a 0.01% (w/v) solution of

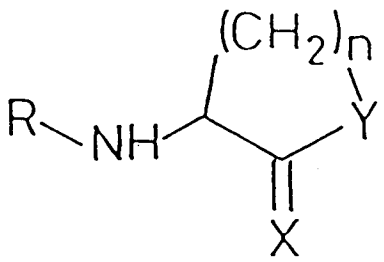
acridine orange for 3-5 minutes at room temperature. Coupons were mounted on a glass microscope slide with a cover clip and imaged using epifluorescent microscopy under oil immersion. Biofilm development was scored as shown in Table 2.

Table 2

24 hour incubation	As column 1 but with an additional 24 hours without additions	As column 1 but with an additional 24 hours with 50μM OOHL	As column 1 but with an additional 24 hours with 50μM ODHL
Bacteria attached in a confluent layer and in a structured manner	Bacteria attached in a confluent layer and in a structured manner	Bacteria attached in a confluent layer and in a structured manner	Bacteria removed or greatly reduced in numbers, with structures significantly affected.

CLAIMS

1. Use of a compound of formula (I)



(I)

wherein: n is 2 or 3;
Y is O, S or NH;
X is O, S or NH;
and R is C_1-C_{18} alkyl or acyl which may be substituted,

in the manufacture of a medicament for the treatment and/or prevention of a bacterial infection in humans or animals by control of colonisation.

2. Use as claimed in claim 1, wherein Y is O, X is O, n is 2 and R is acyl.

3. Use as claimed in claim 1 or claim 2, wherein R carries a keto or hydroxy group.

4. Use as claimed in claim 3, wherein R carries a keto group in the 3-position.

5. Use as claimed in any one of claims 1 to 4, wherein R is a C_8-C_{18} group.

6. Use as claimed in any one of claims 1 to 5, wherein R is a 3-oxododecanoyl group or a 3-oxotetradecanoyl group.

7. Use as claimed in any one of claims 1 to 6, wherein the infection is caused by *Vibrio anguillarum* or *Aeromonas* spp.

8. Use as claimed in any one of claims 1 to 7, wherein the infection causes vibriosis or furunculosis disease in fish.

9. Method of removing a biofilm from a surface which comprises treating the surface with a compound of formula (I) as defined in claim 1.

10. Method as claimed in claim 9, wherein the surface is the inside of an aqueous liquid distribution system.

11. Method as claimed in claim 10 or claim 11, wherein the compound is in solution.

12. Antibacterial composition comprising a compound of formula (I), as defined in claim 1, and a bacteriocidal agent.

13. Article coated and/or impregnated with a compound of formula (I) in order to inhibit and/or prevent biofilm formation thereon.

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/GB 98/03548

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N43/08 A61K31/365 C02F1/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 8516 Derwent Publications Ltd., London, GB; Class B03, AN 85-096666 XP002094722 & JP 60 045568 A (MITSUBISHI GAS CHEM CO INC), 12 March 1985 see abstract & CHEMICAL ABSTRACTS, vol. 103, no. 19, 11 November 1985 Columbus, Ohio, US; abstract no. 160377, see abstract --- -/--	1,2,5,12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	CHEMICAL ABSTRACTS, vol. 125, Columbus, Ohio, US; abstract no. 322642, M.GIVSKOV ET AL.: "Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling" XP002094720 see abstract & J.BACTERIOL., vol. 178, no. 22, 1996, pages 6618-6622, ---	1-13

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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